Physiological responses of the eustigmatophycean *Nannochloropsis salina* to aqueous diesel fuel pollution

OCEANOLOGIA, 47 (1), 2005.
pp. 75–92.

KEYWORDS
Diesel fuel pollution
Physiological responses
Nannochloropsis salina

Nagwa Gamal-El-Din Mohammady[1,*]
Yean-Chang Chen[2]
Abd-El-Ruhman Aly El-Mahdy[3]
Rania Farag Mohammad[4]

[1] Department of Botany, Faculty of Science,
Muharram Beck, Alexandria University, Alexandria, Egypt;

e-mail: nagwa_phyco@yahoo.com

*corresponding author

[2] Department of Aquaculture,
National Taiwan Ocean University, Keelung, Taiwan

[3] Department of Botany, Faculty of Agriculture,
Nile Valley University, Atbara, Sudan

[4] Department of Biology, Faculty of Science,
Omar El-Mokhtar University, El Baidaa, Libya

Received 4 August 2004, revised 12 January 2005, accepted 18 January 2005.

Abstract

The marine eustigmatophycean microalga *Nannochloropsis* (*Monallantus*) *salina* Hibberd was cultivated in a batch culture in the presence of various concentrations (0, 25, 50, 75 and 100%) of an aqueous extract of diesel fuel oil in order to assess the influence of the pollutant on the growth and certain physiological responses of the microalga. The growth data revealed a significant negative effect of the various pollutant concentrations on the algal cell number ($p \leq 0.05$). However, at the mid-logarithmic growth phase (day 8), the algal cells were analysed for chlorophyll *a*, β-glucan, amino acid pool, C/N ratio and elemental composition. According to our results, *N. salina* was significantly affected by the pollution with regard to the different physiological parameters examined, and this significance may be negative, positive or variable. The effect of the pollutant on cellular β-glucan and the total

amount of amino acids was negative; however, the composition of the cellular amino acid pool remained unaffected. A positive effect of the pollutant on cellular chl *a* and the C/N ratio was observed. In addition, the pollutant showed variable effects on the composition of different elements, as shown by energy-dispersive X-ray microanalysis. Also, an existence correlation between different elements was statistically reported.

1. Introduction

Since the marine environment has complex physical, chemical and biological forcing features that affect the native microflora, experiments that assess the effects of petroleum hydrocarbon pollution on naturally occurring phytoplankton assemblages are required in order to understand the relationship between their biology and pollution (Piehler et al. 2003). Petroleum is a significant anthropogenic pollutant in the marine environment (National Research Council 1985) and may affect the community composition of the phytoplankton; these communities are an important source of primary production (Sullivan & Currin 2000). The adverse effects of petroleum pollution on this integral component of coastal ecosystems may be of great significance (Piehler et al. 2003). The effects of petroleum hydrocarbon on microorganisms, including microalgae, have been investigated to address the problem of oil pollution. Russell & Hugo (1988) demonstrated that microorganisms utilise only those hydrocarbon molecules that are dissolved in the aqueous phase. When the microorganisms that can metabolise these compounds are studied, the rate of metabolism sometimes exceeds the rate of mass transfer from the environment to the cell, which leads not only to limitation of growth rate (Volkering et al. 1992) but also to the absence of a toxic effect by these hydrocarbons (Sikkema et al. 1995). A possible explanation for the latter is that the mass transfer of molecules to the cells is limited by the small surface area of the solid particles (Wodziński & Coyle 1974), and the solid hydrocarbon becomes toxic only after long periods of incubation (Sikkema et al. 1995). However, as Gill & Ratledge (1972) demonstrated, the toxicity of alkanes is related to their chain length, which correlates perfectly with their solubility in water and their hydrophobicity.

Diesel fuel was selected as the representative petroleum pollutant because it is common in many coastal waters and has been shown to have an impact on native microorganisms (Piehler et al. 1997). It is a crude oil fraction consisting mostly of linear and branched alkanes with carbon chain lengths of between C10 and C20 (Whyte et al. 1998).

Long-term studies of physiological responses of marine microalgae to pollution have been carried out (Yang et al. 2002). These responses can be assessed by growth measurements (Atlas et al. 1976), metabolic or

photosynthetic activities (Sierra-Alvarez & Lettinga 1991), macromolecules, ATP and adenylate energy (Yang et al. 2002). In addition, X-ray microanalysis of algae is a useful tool in identifying aquatic system contamination and is a good means of assessing the absorbance of elements (Tien 2004). However, the conclusion that many investigators (Morales-Loo & Goutx 1990, Plante-Cuny et al. 1993) have reached is that petroleum hydrocarbons, including diesel fuel, influence microalgal biomass either positively or negatively.

Nannochloropsis is the sole marine genus of the Eustigmatophyceae (Hibberd 1981). It is generally described as a component of the picoeukaryotic plankton since its size ranges from 2–5 μm. (Hu & Gao 2003). Therefore, these organisms are metabolically very active as a result of their favourable surface-to-volume relationship (Moreno-Garrido et al. 1998). Producing an algal biomass by photosynthesis, they thus stand at the beginning of the food chain in aquatic ecosystems. Picoeukaryotic plankton are found throughout the world's oceans at concentrations between 10^2 and 10^4 cells per cm^3 in the upper photic zone (Caron et al. 1999). They play significant roles in the global carbon and mineral cycles, especially in oligotrophic sea waters (Fogg 1995). Furthermore, this microalga is widely used for feeding fish larvae, as it contains highly nutritional compounds such as sterols (Véron et al. 1998) and polyunsaturated fatty acids (Rocha et al. 2003). These fatty acids, especially C20:5ω3, are also the reason for the good growth of fish fry fed on this alga (Zittelli et al. 1999). Previously, this alga used to be known as the 'marine *Chlorella*'; however, it contains no chlorophyll other than chlorophyll *a* (Hibberd 1988). The interest in *Nannochloropsis* as a source of valuable pigments is not related to its capacity for single pigment accumulation, but to the availability of a range of pigments such as zeaxanthin, canthaxanthin and astaxanthin, each with high production levels (Lubián et al. 2000).

The present study thus aims to evaluate the effects of an aqueous diesel fuel pollutant on growth and different physiological processes in the marine picoeukaryotic *Nannochloropsis salina*, an important component of the food chain.

2. Material and methods

Biological material and culture maintenance

The culture material of the eustigmatophyte *Nannochloropsis* (*Monallantus*) *salina* Hibberd was obtained from the Mariculture Centre in Eilat, Israel, and that, in turn, originally from the Solar Energy Research

Institute's (SERI) Culture Collection in Golden, Colorado, USA. The algal material was grown in BES (Boussiba's Enriched Seawater, Boussiba et al. 1987).

Preparation of the aqueous extract of diesel fuel

A stock solution of polluted culture medium was prepared using the method of Boylan & Tripp (1971). BES (1 dm^3) was shaken overnight with diesel fuel (50 cm^3) and the aqueous fraction was recovered using a separating funnel. Aliquots of this aqueous fraction were diluted with BES (control) as appropriate in order to produce culture media containing 25, 50, 75 and 100% of the quantity of pollutant present in the stock solution. In the context of this paper, the BES media so produced are referred to as '25, 50, 75 and 100% polluted medium', respectively. GC analysis of the diesel oil re-extracted from the 100% polluted medium indicated that the main fractions of the pollutant were normal and branched alkanes with 10–21 carbon atoms together with c. 8% aromatic hydrocarbons.

Culture conditions

20 cm^3 of exponentially grown algae were added to 100 cm^3 of the control solution and contaminated BES media in triplicate in 500 cm^3 flasks equipped with inlet and outlet tubes for aeration at an ambient temperature maintained at $25 \pm 1°C$. Cultures were continuously agitated by bubbling with sterile air enriched with CO_2. Illumination was provided by fluorescent lamps with an irradiance of 300 μmol m^{-2} s^{-1}, under a 16 h/8 h light/dark regime.

Measurement of growth

Growth was determined daily by a cell count using a haemocytometer (Fuchs-Rosenthal grid, 0.1 mm deep) under a light microscope (Olympus, Transmission, LM) (Schoen 1988). Growth data was subjected to standard one-way Analysis of Variance (ANOVA) according to Agarwal (1988). All means were tested by the least-squares difference (LSD), where the difference of $p \leq 0.05$ was significant. Least-squares linear regression analysis was applied to evaluate the relationship between the variables.

At the mid-logarithmic growth phase, the algal cells were harvested by centrifuging the cultures at 1000x g for 5 min for the following analyses:

Determination of β-glucan

The glucan was extracted according to Dubois et al. (1956) with 0.05M H_2SO_4 at 60°C for 10 min and analysed by the phenol-sulphuric

acid method. Cellular glucan was determined as the mean of three samples with SD $\pm$ 0.001.

Determination of amino acid composition

The protocol used by Moore et al. (1958) was followed to estimate the total amino acids. According to this method, 0.05 g of each dried algal sample was mixed with 10 cm^3 of 6N HCl containing traces of mercaptoethanol (5 μl per 10 cm^3 acid). The samples were hydrolysed at 110°C for 24 hours. The hydrolysed sample was diluted at 25 cm^3 with distilled water and dried by slow evacuation. The dried residue was dissolved in 0.25M sodium citrate buffer (pH 2.2) and run through a Beckman amino acid analyser. Data from the amino acid pool was subjected to standard two-way Analysis of Variance (ANOVA) according to Agarwal (1988). All means were tested by the least-squares difference (LSD), where a difference of $p \leq 0.05$ was significant.

Estimation of chlorophyll *a*

The extraction and estimation of the chlorophyll *a* concentration were performed according to Sterman (1988). The chlorophyll *a* content was extracted in 90% acetone and estimated spectrophotometrically using a Perkin Elmer spectrophotometer (Lambadal), applying the following equation:

$$\text{Chlorophyll } a = 11.93\ A_{664} - 1.93\ A_{647} \qquad [\text{mg chl } a\ (\text{cm})^{-3}].$$

Chlorophyll *a* data was subjected to standard one-way ANOVA according to Agarwal (1988). All means were tested by LSD, where the difference of $p \leq 0.05$ was significant.

Estimation of Carbon and Nitrogen

These were determined using the elemental analyser PE2400 Series II CHNS/O. The C/N ratio was determined as the mean of three samples with SD $\pm$ 0.1.

Scanning X-ray microanalysis

The relative concentrations of the element composition were analysed using energy-dispersive X-ray microanalysis (JGOL JSM-5300 scanning microscopy X-ray OXFORD). X-ray data were subjected to standard one-way ANOVA according to Agarwal (1988). However, the similarity matrices between elements were produced using the Czekanowski (1913) similarity coefficient.

3. Results

The growth data of *N. salina* expressed as a cell number revealed a significant negative effect of the pollutant on the algal cells ($p \leq 0.05$). The maximum cell number was 18×10^6 per cm^3 culture on the 13th day in control cultures, while in the 25, 50, 75 and 100% diesel treated cells, the maximum cell number gradually fell to 12.3×10^6, 7.8×10^6, 4.2×10^6 and 3.9×10^6 per cm^3 culture respectively (Fig. 1).

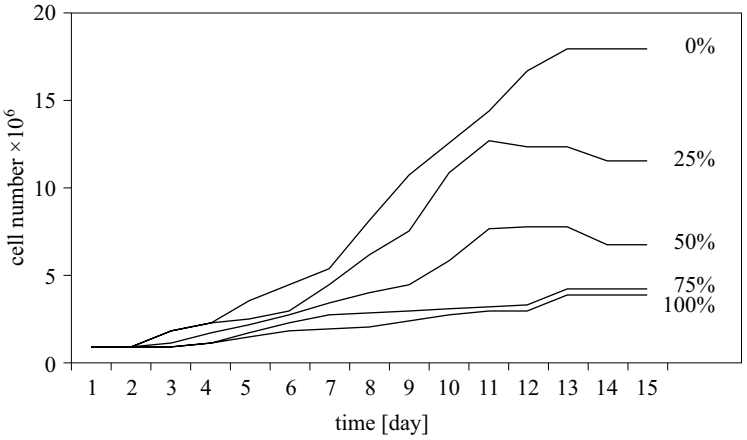

Fig. 1. *Nannochloropsis salina* growing in the control and various concentrations of aqueous diesel fuel extract

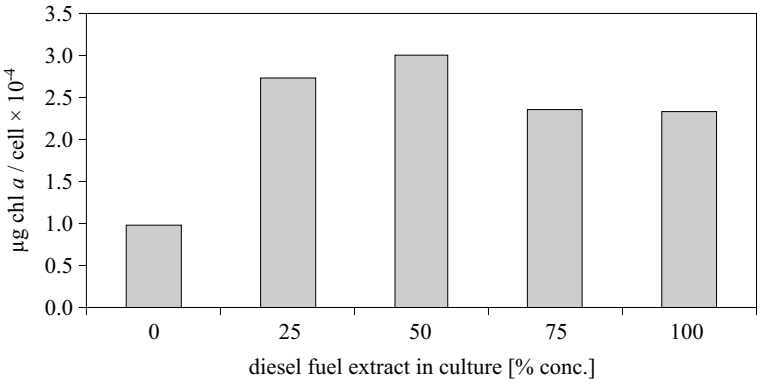

Fig. 2. Chlorophyll *a* content of *Nannochloropsis salina* growing in the control and various concentrations of aqueous diesel fuel extract

The data in Fig. 2 show the positive significant effect of the pollutant on cellular chlorophyll *a*: its concentration increased gradually from

0.97×10^{-4} μg per cell in the control culture to reach a maximum value of 3×10^{-4} μg chl a per cell in 50% aqueous diesel treated cells followed by a decrease to 2.33×10^{-4} μg chl a per cell in 100% aqueous diesel treated cells.

Our data on cellular β-glucan demonstrated the significant negative effect of the pollutant. Fig. 3 shows that the concentration of β-glucan dropped from 3.6×10^{-6} μg per cell in the control culture to 1.4×10^{-6} μg per cell in 100% aqueous diesel treated cells.

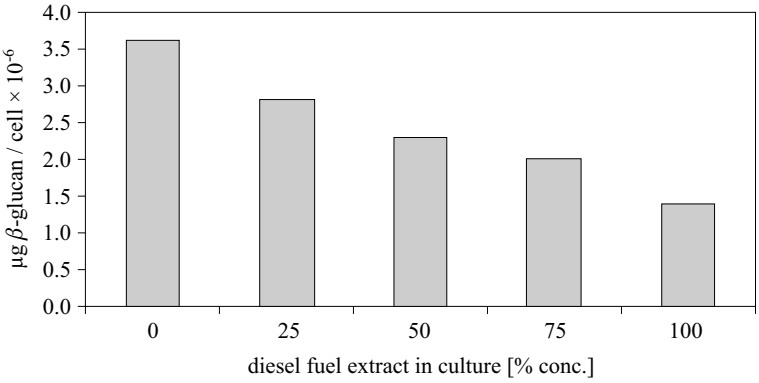

Fig. 3. β-glucan content of *Nannochloropsis salina* growing in the control and various concentrations of aqueous diesel fuel extract

Table 1 shows the presence of seventeen amino acids in *N. salina*, nine of which are essential components. Statistical analysis by two-way ANOVA indicated a significant negative effect of the pollutant on the amount of cellular amino acids ($p \leq 0.05$). However, the composition remained unaffected. Kreb's cycle amino acids decreased gradually to a minimum value (12.2 mg per 100 g fresh weight); the same trend was detected in the triose and pyruvate pathway amino acid families, as well as the Shikimic acid family.

The C/N ratio increased slightly (Table 2) from 5.5 in the control culture to 6.2 in the 50% aqueous diesel polluted culture, and then fell somewhat to reach 5.55 in 100% aqueous diesel polluted cells.

As revealed by energy-dispersive X-ray microanalysis Table 3, the investigated alga did not contain any elements other than those comprising the nutrient medium. A relatively large quantity of S followed by successively smaller amounts of P and Cl were detected in the control culture. The concentration of elements in the algal cells (Table 3) varied according to the pollutant concentration applied. However, a marked increase in S concentration was observed in the 25% polluted cells, since S is a significant component of diesel fuel; even though this value decreased

Table 1. Total amino acid composition (mg per 100 g f.w.) of *Nannochloropsis salina* growing in the control and various concentrations of aqueous diesel fuel extract

	Krebs cycle family											
	Glutamate family						Aspartate family					
	Glutamate	Arginin*	Proline	Histidine*	Total	Total	Aspartic	Lysine*	Isoleucine*	Methionine*	Total	Total
0%	1.98	1.48	3.99	0.75	8.20	3.58	1.59	1.14	2.39	0.44	9.14	17.34
25%	1.53	1.23	3.57	0.67	7.00	3.29	1.18	0.97	1.61	0.43	7.48	14.48
50%	1.53	1.11	3.52	0.62	6.78	2.49	0.95	0.67	1.40	0.41	5.92	12.70
75%	1.25	1.00	2.80	0.60	5.65	2.22	0.82	0.24	1.31	0.37	4.96	10.61
100%	1.21	1.00	2.22	0.52	4.95	2.20	0.74	0.09	0.97	0.28	4.28	9.23

	Triose-puruvic acid family									Shikimic acid family			
	Triose family				Puruvate family								
	Glycine	Serine	Cysteine	Total	Alanine	Valine*	Leucine*	Total	Total	Phenyl. alanine*	Tyrosine	Total	Total
0%	1.21	1.22	0.03	2.46	3.05	1.59	2.42	7.06	9.52	0.95	0.47	1.42	28.28
25%	1.09	0.92	0.04	2.05	2.48	1.25	1.76	5.49	7.54	0.78	0.39	1.17	23.19
50%	1.04	0.86	0.04	1.94	2.32	0.98	1.73	5.03	6.97	0.59	0.38	0.97	20.64
75%	0.91	0.72	0.03	1.66	1.90	0.90	1.67	4.47	6.11	0.41	0.35	0.76	17.48
100%	0.15	0.62	0.03	0.80	1.37	0.32	1.59	3.28	4.08	0.38	0.31	0.69	14.00

* Represents essential amino acids.

Table 2. C/N ratio in *Nannochloropsis salina* growing in the control and various concentrations of aqueous diesel fuel extract. (Data expressed as percentages)

	Diesel fuel concentrations				
	0%	25%	50%	75%	100%
C/N ratio*	5.50	5.97	6.20	5.62	5.55

* Each ratio is the mean of three measurements with SD $\pm$ 0.1.

gradually with increasing pollutant concentration, it was still higher than the control value. On the other hand, a dramatic drop in Cl was observed during the treatments. Na, Mg, Br, P and K contents varied according

Table 3. X-ray micro-analysis of the elemental composition of *Nannochloropsis salina* growing in the control and various concentrations of aqueous diesel fuel extract. (Data expressed as percentages)

	Elements										
	Na	Mg	Br	P	S	Cl	K	Ca	Fe	Cu	Zn
0%	8.45	8.44	2.01	19.43	22.68	16.83	2.88	8.44	1.33	5.46	4.05
25%	11.34	16.73	1.89	22.24	36.40	0.49	8.50	2.86	0.33	5.67	4.89
50%	10.14	10.54	13.48	14.53	31.65	0.47	6.41	0.22	0.38	7.10	5.66
75%	12.95	16.08	5.73	16.47	32.01	1.66	14.74	6.68	0.23	5.86	5.55
100%	8.72	14.48	11.05	20.21	28.97	0.65	4.12	0.70	0.01	3.74	2.89

to the pollutant concentration applied. Nevertheless, the Fe concentration decreased gradually with rising pollutant concentration. However, both Cu and Zn rose gradually to 50% pollutant, then decreased to reach their minimum values at 100% diesel concentration. Statistical analysis by one-way ANOVA indicated that the effect of the pollutant on the elements was significant ($p \leq 0.05$). The similarity matrices showed a significant correlation (positive or negative) to exist between different elements in relation to the pollutant concentration applied. A highly positive correlation was detected between Na and K (0.98), followed by Cl and Fe (0.93), and then Zn and Cu (0.85). In contrast, a highly negative correlation was

Table 4. Similarity matrices of the elemental composition of *Nannochloropsis salina* growing in the control and various concentrations of aqueous diesel fuel extract

	Na	Mg	Br	P	S	Cl	K	Ca	Fe	Cu	Zn
Na	1.0										
Mg	0.69	1.0									
Br	–0.17	–0.13	1.0								
P	–0.23	0.35	–0.61	1.0							
S	0.71	0.79	0.08	0.07	1.0						
Cl	–0.51	–0.72	–0.52	0.13	–0.85	1.0					
K	0.98	0.66	–0.12	–0.29	0.60	–0.48	1.0				
Ca	0.12	–0.22	–0.75	0.08	–0.5	0.75	0.18	1.0			
Fe	–0.67	–0.81	–0.42	0.18	–0.78	0.93	–0.68	0.52	1.0		
Cu	–0.52	–0.47	0.56	–0.19	0.02	–0.13	–0.61	–0.69	–0.18	1.0	
Zn	–0.58	–0.14	0.57	0.14	0.12	–0.33	–0.65	–0.85	–0.28	0.85	1.0

detected between Zn and Ca, and S and Cl (–0.85), and then btween Fe and Mg (–0.81). The similarity matrices between the elements analysed are reported in Table 4.

4. Discussion

By means of the statistical analysis, our data have highlighted significant physiological responses of the investigated alga as expressed by compositional changes, which may be positive, negative or variable. The growth of *N. salina* was negatively affected by the pollutant since the cell number gradually fell with increasing concentrations of the aqueous diesel extract to less than a quarter of the initial number in 100% treated cells relative to the control. Growth reduction by oil pollution, including diesel fuel, has been demonstrated in many algal species, e.g. *Scenedesmus quadricauda* (Dennington et al. 1975), *Isochrysis* sp. (Ansari et al. 1997) and *Tetraselmis suecica* (Fabregas et al. 1984). According to Ikawa et al. (1992), growth reduction results from the inhibition of enzyme systems, photosynthesis, respiration, protein and nucleic acid synthesis. On the other hand, Piehler et al. (2003) considered that the reduction of algal growth resulting from diesel spills might be due to inhibition of cell division. Lai & Khanna (1996) and Tongpim & Pickard (1996) attributed growth inhibition in *Acinetobacter calcoaceticus* and *Rhodococcus* sp., cultivated in the presence of the diesel fuel metabolic intermediates octadecane and anthracene, to their reduced bioavailability to the strains. Our unpublished data indicated a marked reduction in *N. salina* cell viability with increasing amounts of added fuel extract although the pollutant composition, monitored during the experimental culture, did not change. That is why the growth trend of the cells in different polluted cultures was similar in our work, although a broad difference in the cell number was observed. The reduction in algal cell number may be accompanied by delayed cell division: this delay is proportional to the concentration of pollutant added, which in turn is a function of the decrease in algal cell bioavailability under these conditions of stress.

A positive effect of the pollutant was obviously shown on the total yield production of chlorophyll *a*. The amount of chlorophyll *a* increased gradually to reach a maximum value in the cells treated with 50% aqueous diesel extract concentration, then decreased to reach more than twice the amount produced by control culture in 100% treated cells. Nechev et al. (2002) postulated that diesel fuel causes a disruption of the optimal physical state of the cytoplasmic membranes, thus raising the permeability of these membranes, which in turn facilitate the entry of diesel fuel into the cells and the accumulation of a high quantity of hydrocarbons. This

accumulation of aqueous diesel fuel causes swelling of *N. salina* cells (unpublished data), the original volume of which may double; this could also be the reason why chl *a* production also doubles. However, since diesel fuels are hydrophobic compounds (National Research Council 1985), problems with their transport may delay or prevent any potentially toxic impact on microalgae (Piehler et al. 2003), and hence an excessive increase in pigment production (Sikkema et al. 1995). The same result was obtained by Wang et al. (2002), who found that a concentration up to 6 μg dm^{-3} of anthracene when introduced to *Isochrysis galbana* and *Skeletonema costatum* stimulates chlorophyll *a*, carotenoid content, and superoxide dismutase activity.

Cellular carbohydrates vary quantitatively according to culture conditions (Brown et al. 1998). Carbohydrates are the first products of photosynthesis in all algae (Calvin-Benson cycle) and provide the precursors for all cell components. The principal storage polysaccharide in the Eustigmatophyceae (including *N. salina*) is β-glucan. This is known as chrysolaminaran in the Chrysophyceae, Eustigmatophyceae, Haptophyceae (Prymnesiophyceae) and Xanthophyceae, as paramylon in the Euglenophyceae, and as laminaran in the Phaeophyceae (Granum & Myklestad 2001). This glucan can be readily hydrolysed by exo-β-1,3-glucanase, in that glucose is split off at the non-reducing end (Myklestad et al. 1982). In contrast, glucose can be metabolised by the respiratory pathways, providing precursors for amino acid biosynthesis (Granum & Myklestad 1999). By supplying essential carbon skeletons for amino acid biosynthesis, β-glucan also furnishes energy and reducing power to the cell. Both energy (ATP) and an electron donor (NADPH) are required for reductive biosynthesis to occur. Further, β-glucan provides precursors for the synthesis of other polysaccharides and/or nucleic acids (Granum & Myklestad 2001). The present investigation confirmed that β-glucan was reduced by aqueous diesel fuel extract and that the reduction was found proportional to the increase in pollutant concentration. However, the cellular quantity of β-glucan in 100% treated cells was less than half of that in the control. Since stressed β-glucan indirectly reflects the behaviour of different cellular metabolites, we can postulate that both photosystem I and photosystem II yield products that will change in the stressed cells of *N. salina*. As demonstrated by Torzillo (1998), environmental stress affects the functioning of photosystem II (PS II) in *Spirulina* directly or indirectly, and the changes in the alga's biomass yield correlate well with changes in the daily integrated value of the estimated electron transport rate (ETR) through the PS II. ETR has proved to be a simple and reliable parameter for estimating the photosynthetic performance of *Spirulina*. In addition, petroleum hydrocarbons have been found to increase membrane permeability, which can lead to reduced proton

motive force in the cell (Sikkema et al. 1995). If the proton pumps do not perform effectively, as discussed by Piehler et al. (2003), compromising the electrochemical gradient across the thylakoid membrane results in a decrease in the photosynthetic yield of the two photosystems.

The present study has indicated a reduction effect of the pollutant on the cellular amino acid pool of *N. salina*, although the individual amino acid composition was itself not affected. Previous studies have shown that cellular amino acids are affected by external environment stress (Caldwell 1995, Barrett & Elmore 1998, El-Sheekh 2000). The reduction effect of pollution by aqueous diesel fuel on the amounts of different amino acids may be an indirect reflection of the reduction in β-glucan content.

Under batch culture conditions, the chemical composition of microalgae may vary, largely as a complex response to environmental conditions. Carman et al. (1997) found that hydrocarbon contamination enhanced nitrogen availability. Other investigators (Burkhardt et al. 1999, Riebesell et al. 2000) demonstrated that the C/N ratio was influenced by a carbon-enriched culture medium. In contrast, Chabbi & Rumpel (2004) considered that C/N ratios are a consequence of the presence of decomposing plant and/or microbial (including algae) residues. In this connection, our results regarding C/N ratios were slightly elevated relative to the control up to 50% aqueous diesel extract concentration. After that, the ratio decreased again to approximately the control value, a result indicative of the constant proportions of the two elements despite the addition of the pollutant. Further, the trend in the C/N ratio seems to be roughly parallel to that of chlorophyll *a* production during the experimental culture. This result is in agreement with Rebolloso-Fuentes et al. (1999), who found that elemental carbon varied in parallel with the pigment content in microalgae.

The significant role of individual elements in the algal cell has been reiterated by many authors (DeBoer 1981, Speransky et al. 1999, Sánchez-Rodríguez et al. 2001). The elemental abundance in algae is apparently controlled by the elemental abundance in the medium, whereas metabolic processes as well as environmental factors relevant to the habitat modify the final concentration of a given element in the algal cell (Sánchez-Rodríguez et al. 2001). In the present work, X-ray microanalysis of elements showed that S was the most abundant of the elements detected, since S is a major constituent of diesel fuel. Further, there were no elements other than those making up the nutrient medium, a result indicating that diesel fuel pollutant contains no metal that can affect the cellular metabolism of *N. salina* and that the hydrocarbon pollutant is the only effective factor. However, our data showed a variable significant effect of the pollutant ($p \leq 0.05$) on the element concentrations. Moreover, there was an obvious

increase in the concentrations of some positively charged ions following the addition of different aqueous diesel extract concentrations. As explained by Sikkema et al. (1995), the stimulation of the leakage of protons and potassium ions across various membranes after the addition of lipophilic compounds may be accompanied by osmotic disturbance and/or correlated with a specific mechanism. Kang et al. (2003) postulated that changes in the ratio of peak intensities of X-ray spectra of some elements, relative to the environmental pollution, reflect antagonism between elements in algae. However, in the present work, similarity matrices reveal an existence correlation between certain elements: some have positive, others have negative existence correlations. The significant, highly negative correlation between Na and Cl as well as that between Cl and K indicate a disturbance of the osmotic balance in the algal cell. As postulated by Speransky et al. (1999), Cl and K are two osmotically important ions. Further, Na and Cl are involved in charge-balancing mechanisms.

5. Conclusions

- The influence of aqueous diesel fuel pollution on *N. salina* induced a decrease in cell bioavailability leading to delayed cell division and hence, a decrease in the cell number that is proportional to the pollutant concentration.
- The physiological behaviour of *N. salina* is significantly affected by the pollutant. The effect may be positive, negative or variable.
- Diesel fuel is a hydrophobic compound and, when applied to *N. salina*, disrupts the optimal physical state of cytoplasmic membranes, thus disturbing the osmotic balance of the algal cell. Accordingly, cell permeability increases, which in turn stimulates the influx of the pollutant and probably the accumulation of a high quantity of hydrocarbons, which causes cell swelling. As a result, the pollutant has a positive significant effect on cellular chlorophyll *a* production, although the C/N ratio rises only slightly during the experimental culture.
- On the other hand, increased membrane permeability in microalgae reduces the proton motive force in the cell, which in turn cooperates with the electrochemical gradient across the thylakoid membranes resulting in a decrease in the photosynthetic yield of photosystems I and II. Accordingly, there is a significant negative effect of the pollutant on β-glucan, and on the overall cellular amino acid pool.

- Finally, the significant variable effect of the pollutant on the elemental concentrations in the investigated alga may be accompanied by osmotic disturbance and/or be correlated to a specific mechanism.

References

Agarwal B. L., 1988, *Basic statistics*, Wiley Eastern Ltd., New Delhi, 748 pp.

Ansari Z. A., Saldanha M. C., Rajkumar R., 1997, *Effects of petroleum hydrocarbons on the growth of microalga Isicrysis sp. (Chrysophyta)*, Indian J. Mar. Sci., 26, 372–376.

Atlas R. M., Schofield E. A., Morelli F. A., Cameron R. E., 1976, *Effects of petroleum pollutants on Arctic microbial populations*, Environ. Pollut., 10, 35–45.

Barrett G. C., Elmore D. T., 1998, *Amino acids and peptides*, Cambridge Univ. Press, 240 pp.

Boussiba S., Vonshak A., Cohen Z., Avissar Y., Richmond A., 1987, *Lipid and biomass production by halotolerant microalga Nannochloropsis salina*, Biomass, 12, 17–24.

Boylan D. B., Tripp B. W., 1971, *Determination of hydrocarbons in seawater extracts of crude oil and crude oil fractions*, Nature, 230 (5288), 44–47.

Brown M. R., McCausland M. A., Kowalski K., 1998, *The nutritional value of four Australian microalgal strains fed to Pacific oyster Crassostrea gigas spat*, Aquaculture, 165 (3), 281–293.

Burkhardt S., Zondervan I., Riebesell U., 1999, *Effects of CO_2 concentration on C:N:P ratio in marine phytoplankton: a species comparison*, Limnol. Oceanogr., 44 (3), 683–690.

Caldwell D. R., 1995, *The effects of environmental factors on microbes*, [in:] *Microbial physiology and metabolism*, Wm. C. Brown Publ., Dubuque, IA, 353 pp.

Carman K. R., Fleeger J. W., Pomarico S. M., 1997, *Response of benthic food web to hydrocarbon contamination*, Limnol. Oceanogr., 42 (3), 561–571.

Caron D. A., Peele E. R., Lim E. L., Dennett M. R., 1999, *Picoplankton and nanoplankton and their trophic coupling in the surface waters of the Sargasso Sea south of Bermuda*, Limnol. Oceanogr., 44 (2), 259–272.

Chabbi A., Rumpel C., 2004, *Chemical composition of organic matter in extremely acid, lignite-containing lake sediments impacted by fly ash contamination*, J. Environ. Qual., 33 (2), 628–636.

Czekanowski J., 1913, *Zarys metod statystycznych*, Anthropol. Anz., 9, 227–249.

DeBoer J. A., 1981, *Nutrients*, [in:] *The biology of seaweeds*, C. S. Lobban & M. J. Wynne (eds.), Blackwell Sci. Publ., Oxford, 356–392.

Dennington V. N., George H. M., Wybron C. H. E., 1975, *The effect of oil on growth of fresh water phytoplankton*, Environ. Pollut., 8, 233–237.

Dubois M., Gilles K. A., Hamilton J. K., Rebers P. A., Smith F., 1956, *Colorimetric method for determination of sugar and related substances*, Anal. Chem., 28, 350–356.

El-Sheekh M. M., 2000, *Effect of glyphosate herbicide on growth, photosynthesis and some metabolic activities on the green alga Chlorella kessleri (Chlorophyta)*, Egypt. J. Phycol., 1, 87–97.

Fabregas J., Herrero C., Veiga M., 1984, *Effect of oil and dispersant on growth and chlorophyll a content of the marine microalga Tetraselmis suecica*, Appl. Environ. Microbiol., 47 (2), 445–447.

Fogg G. E., 1995, *Some comments on picoplankton and its importance in the pelagic ecosystem*, Aquat. Microb. Ecol., 9 (1), 33–39.

Gill C. O., Ratledge C., 1972, *Toxicity of n-alkanes, n-alk-1-enes, n-alkan-1-ols and n-alkyl-1-bromides towards yeasts*, J. Gen. Microbiol., 72, 165–172.

Granum E., Myklestad S. M., 1999, *Effect of NH_4 assimilation on dark carbon fixation and β-1,3-glucan metabolism in the marine diatom Skeletonema costatum (Bacillariophyceae)*, J. Phycol., 35, 1191–1199.

Granum E., Myklestad S. M., 2001, *Mobilization of β-1,3-glucan and biosynthesis of amino acids induced by NH_4^+ addition to N-limited cells of the marine diatoms Skeletonema costatum (Bacillariophyceae)*, J. Phycol., 37 (5), 772–782.

Hibberd D. J., 1981, *Notes on the taxonomy and nomenclature of the algal classes Eustigmatophyceae and Tribophyceae (synonym Xanthophyceae)*, Bot. J. Linn. Soc., 82 (2), 93–119.

Hibberd D. J., 1988, *Eustigmatophyceae*, [in:] *Handbook of Protoctista*, L. Margulis, J. Corliss, M. Melkonian & D. Chapman (eds.), Jones & Bartlett Publ., Boston, 30–50.

Hu H., Gao K., 2003, *Optimization of growth and fatty acid composition of a unicellular marine picoplankton, Nannochloropsis sp., with enriched carbon sources*, Biotechnol. Lett., 25 (5), 421–425.

Ikawa M., Mosley S. P., Barbero L. J., 1992, *Inhibitory effects of terpene alcohols and aldehydes on growth of green alga Chlorella pyrenoidosa*, J. Chem. Ecol., 18 (10), 1755–1760.

Kang S. X., Shen X. S., Huang Y. Y., Ju X., Wu Z. Q., 2003, *SR-XRF analysis of characteristics of heavy element concentration in qingdao algae and application to monitoring oceanic pollution*, Sci. Total Environ., 23 (1), 94–97.

Lai B., Khanna S., 1996, *Mineralization of ^{14}C octacosane by Acinetobacter calcoaceticus S30*, Can. J. Microbiol., 42, 1225–1231.

Lubián L. M., Montero O., Moreno-Garrido I., Huertas E., Sobrino C., González -Delvalle M., Parés G., 2000, *Nannochloropsis (Eustigmatophyceae) as a source of commercially valuable pigments*, J. Appl. Phycol., 12 (3)–(5), 249–255.

Moore S., Spackman D. H., Stein W., 1958, *Chromatography of amino acids on sulphonated polystyrene resins*, Anal. Chem., 30, 1185–1190.

Morales-Loo M. R., Goutx M., 1990, *Effects of water soluble fractions of the Mexican crude oil 'Isthmus Cactus' on growth, cellular content of chlorophyll a, and lipid composition of planktonic microalgae*, Mar. Biol., 104, 503–509.

Moreno-Garrido I., Blasco J., González-Delvalle M., Lubián L. M., 1998, *Differences in Copper accumulation by the marine microalga Nannochloropsis gaditana, submitted to two different thermal treatments*, Ecotox. Environ. Rest., 1 (1), 43–47.

Myklestad S., Djurhuus R., Mohus A., 1982, *Demonstration of exo- (β-1,3)-D-glucanase activity in some planktonic diatoms*, J. Exp. Mar. Biol. Ecol., 56, 205–211.

National Research Council, 1985, *Oil in the Sea: inputs, fates and effects*, Natl. Acad. Press, Washington, DC, 601 pp.

Nechev J. T., Khotimchenko S. V., Ivanova A. P., Stefanov K. L., Dimitrova-Konaklieva S. D., Andreev S., Popov S. S., 2002, *Effect of diesel fuel pollution on the lipid composition of some wide-spread Black Sea algae and invertebrates*, Z. Natürforsch, 57 (C), 339–343.

Piehler M. F., Swistak J. G., Pinckney J. L., Paerl H. W., 1997, *Sub-lethal effects of coastal petroleum pollution on Spartina alterniflora stem epiphytes*, Chemosphere, 35 (11), 2665–2674.

Piehler M. F., Winkelmann V., Twomey L. J., Hall N. S., Currin C. A., Paerl H. W., 2003, *Impact of diesel fuel exposure on the microphytobenthic community of an intertidal sand flat*, J. Exp. Mar. Biol. Ecol., 297, 219–237.

Plante-Cuny M. R., Salen-Picard C., Grenz C., Plante R., Alliot E., Barranguet C., 1993, *Experimental field study of the effect of crude oil, drill cutting and natural biodeposits on microphyto-and macrozoobenthic communities in a Mediterranean area*, Mar. Biol., 117, 355–366.

Rebolloso-Fuentes M. M., Garciá-Sánchez J. L., Fernández-Seville J. M., Acién-Fernández F. G., Sánchez-Pérez J. A., Molina-Grima E., 1999, *Outdoor continuous culture of Porphyridium cruentum in a tubular photobioreactor: quantitative analysis of the daily cyclic variation of culture parameters*, J. Biotechnol., 70 (1)–(3), 271–288.

Riebesell U., Revill A. T., Holdsworth D. G., Volkman J. K., 2000, *The effect of varying CO_2 concentration on lipid composition and carbon isotope fractionation in Emiliania huxleyi*, Geochim. Cosmochim. Acta, 64 (24), 4179–4192.

Rocha J. M., Gracia J. E., Henriques M. H., 2003, *Growth aspects of the marine microalga Nannochloropsis gaditana*, Biomol. Eng., 20 (4)–(6), 237–242.

Russell A. D., Hugo W. B., 1988, *Perturbation of homeostatic mechanisms in bacteria by pharmaceutical*, [in:] *Homeostatic mechanisms in micro-organisms*, R. Whittenbury, G. W. Gould, J. G. Banks & R. G. Board (eds.), Bath Univ. Press, Bath, 206–219.

Sánchez-Rodríguez I., Huerta-Diaz M. A., Choumiline E., Holguín-Quiñones O., Zertuche-González J. A., 2001, *Elemental concentrations in different species of seaweeds from Loreto Bay, Baja California Sur, Mexico: implications for the geochemical control of metals in algal tissue*, Environ. Pollut., 114 (2), 145 –160.

Schoen S., 1988, *Cell counting*, [in:] *Experimental phycology: a laboratory manual*, C. S. Lobban, D. J. Chapman & B. P. Kremer (eds.), Cambridge Univ. Press, New York, 16–22.

Sierra-Alvarez R., Lettinga G., 1991, *The effect of aromatic structure on the inhibition of acetoclastic methanogenesis in granular sludge*, Appl. Microbiol. Biotechnol., 34 (4), 544–550.

Sikkema J., de Bont J. A. M., Poolman B., 1995, *Mechanisms of membrane toxicity of hydrocarbons*, Microbiol. Rev., 59 (2), 201–222.

Speransky V. V., Speransky S. R., Brawley S. H., 1999, *Cryoanalytical studies of freezing damage and recovery in Fucus vesiculosus (Phaeophyceae)*, J. Phycol., 35, 1264–1275.

Sterman N. T., 1988, *Spectrophotometric and fluorometric chlorophyll analysis*, [in:] *Experimental phycology: a laboratory manual*, C. S. Lobban, D. J. Chapman & B. P. Kremer (eds.), Cambridge Univ. Press, New York, 35–46.

Sullivan M. J., Currin C. A., 2000, *Community structure and functional dynamics of benthic microalgae in salt marshes*, [in:] *Concepts and controversies in tidal marsh ecology*, M. P. Weinstein & D. A. Kreeger (eds.), Kluwer Acad. Publ., Dordrecht, 81–106.

Tien C. J., 2004, *Some aspects of water quality in a polluted lowland river in relation to the intercellular chemical levels in planktonic and epilithic diatoms*, Water Res., 38 (7), 1779–1790.

Tongpim S., Pickard M. A., 1996, *Growth of Rhodococcus S1 on anthracene*, Can. J. Microbiol., 42 (3), 289–294.

Torzillo G., 1998, *Optimization of microalgal productivity outdoors: use of an on-line chlorophyll fluorescence technique*, 52nd Ann. Meet. Phycol. Soc. Am.

Véron B., Dauguet J. C., Billard C., 1998, *Sterolic biomarkers in marine phytoplankton. II. Free and conjugated sterols of seven species used in mariculture*, J. Phycol., 34, 273–279.

Volkering F., Breure A. M., Sterkenburg A., van Andel J. G., 1992, *Microbial degradation of polycyclic aromatic hydrocarbons: effect of substrate availability on bacterial growth kinetics*, Appl. Microbiol. Biot., 36, 548–552.

Wang Y., Tang X., Li Y., Liu Y., 2002, *Stimulation effects of anthracene on marine microalgae growth*, Sci. Total Environ., 13 (3), 343–346.

Whyte L. G., Hawari J., Zhou E., Bourbonniére L., Inniss W. E., Greer C. W., 1998, *Biodegradation of variable-chain length alkanes at low temperatures by a psychrotrophic Rhodococcus sp.*, Appl. Environ. Microbiol., 64 (7), 2578 –2584.

Wodziński R. S., Coyle J. E., 1974, *Physical state of phenanthrene for utilization by bacteria*, Appl. Micobiol., 27 (6), 1081–1084.

Yang S., Wu R. S., Kong R. Y., 2002, *Physiological and cytological responses of the marine diatom Skeletonema costatum to 2, 4-dichlorophenol*, Aquat. Toxicol., 60 (1)–(2), 33–41.

Zittelli G. C., Lavista F., Bastianini A., Rodolfi L., Vincenzini M., Tredici M. R., 1999, *Production of eicosapentaenoic acid by Nannochloropsis sp. cultures in outdoor tubular photobioreactors*, J. Biotechnol., 70, 299–312.